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At the Cutting Edge

G protein abnormalities in pituitary adenomas

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Abstract

It has been demonstrated that the majority of secreting and nonsecreting adenomas is monoclonal in origin suggesting that these neoplasia arise from the replication of a single mutated cell, in which growth advantage results from either activation of protooncogenes or inactivation of antioncogenes. Although a large number of genes has been screened for mutations, only few genetic abnormalities have been found in pituitary tumors such as allelic deletion of chromosome 11q13 where the MEN-1 gene has been localised, and mutations in the gene encoding the α subunit of the stimulatory Gs and Gi2 protein. These mutations constitutively activate the α subunit of the Gs and Gi2 protein by inhibiting their intrinsic GTPase activity. Both Gsα and Gi2α can be considered products of protooncogenes (*gsp* and *gip2*, respectively) since gain of function mutations that activate mitogenic signals have been recognized in human tumors. *Gsp* oncogene is found in 30–40% of GH-secreting adenomas, in a low percentage of nonfunctioning and ACTH-secreting pituitary adenomas, in toxic thyroid adenomas and differentiated thyroid carcinomas. The same mutations, occurred early in embriogenesis, have been also identified in tissues from patients affected with the McCune Albright syndrome. These mutations result in an increased cAMP production and in the subsequent overactivation of specific pathways involved in both cell growth and specific programmes of cell differentiation. By consequence, the endocrine tumors expressing *gsp* oncogene retain differentiated functions. The *gip2* oncogene has been identified in about 10% of nonfunctioning pituitary adenomas, in tumors of the ovary and the adrenal cortex. However, it remains to be established whether Gi proteins activate mitogenic signals in pituitary cells. Since Gi proteins are involved in mediating the effect of inhibitory neurohormones on intracellular effectors, it has been proposed that in pituitary tumors the low expression of these proteins, particularly Gi1-3α, may contribute to uncontrolled pituitary cells growth by preventing the transduction of inhibitory signals. While by in vitro mutagenesis it has been demonstrated that activated mutant of Gqα, G12α, G13α and Gzα are fully oncogenic, it remains to be proved whether or not these abnormalities might naturally occur in human tumors and, in particular, in pituitary adenomas.

Keywords: Pituitary adenomas; G proteins; Gsp; Phosphodiesterase

1. Introduction

Pituitary adenomas are mostly benign neoplasia, arising, with variable frequency, from differentiated anterior pituitary cell types. These tumors may either secrete pituitary hormones, causing endocrinological syndromes due to hormone excess, or be functionally silent. Although our understanding of the molecular mechanisms responsible for pituitary tumor formation and progression is still incomplete, during the last few years important new information on the genetic alterations occurring in tumoral pituitary cells has been accumulating. In particular, by X-chromosome inactivation analysis in female patients heterozygous for variant alleles of X-linked genes, it was demonstrated that the majority of secreting and nonsecreting adenomas is monoclonal in origin (Alexander et al., 1990; Herman

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et al., 1990). Thus, these neoplasia arise from the replication of a single mutated cell, in which growth advantage results from either activation of protooncogenes or inactivation of antioncogenes. Although a large number of genes has been screened for mutations, only few genetic abnormalities have reproducibly been found in pituitary tumors (Spada et al., 1994; Shimon and Melmed, 1997). These abnormalities include allelic deletion of chromosome 11q13 where the MEN-1 gene, termed menin, is located (Thakker et al., 1993; Chandrasekharappa et al., 1997; Zhuang et al., 1997), and mutations in the gene encoding the α subunit of the stimulatory G protein (Gsα) (Vallar et al., 1987; Landis et al., 1989; Lyons et al. 1990). While the role of menin as tumor suppressor gene is still unknown, Gsα is a key element for the activation of cAMP dependent pathway, that in pituitary cells is a signal for differentiation and proliferation.

2. G protein structure and function

Gsα belongs to the large family of trimeric G proteins that relay signals from plasma membrane receptors for hormones and neurotransmitters to intracellular effectors (Dhanasekaran et al., 1995; van Biesen et al., 1996; Spiegel, 1996) (Table 1). Each G protein is composed of a guanine nucleotide-binding α subunit and a dimeric subunit that includes a β- and a γ-chain. In resting conditions, the GDP-bound α subunit binds tightly to $\beta\gamma$ and is inactive whereas the GTP-bound form dissociates from the dimer and serves as a regulator of effector proteins. The guanine nucleotide pocket of the α subunit consists of distinct, highly conserved stretches. Critical regions in the C terminus of Gα are involved in specifying the α subunit binding to its respective receptor. The receptor-triggered structural changes cause the release of GDP and the binding of GTP to the open nucleotide-binding cleft. GTP-binding induces a conformational change of the α subunit, leading to a decreased affinity for both the receptor and the $\beta\gamma$-dimer and an increased affinity for a specific intracellular effector. The Gα surface loops are implicated in effector binding and activation (Fig. 1).

The duration of subunit separation is timed by the rate of GTP hydrolysis, a turn-off mechanism inherent in all α subunits. There are two features of the crystal structure of the α subunit that seem to be relevant to GTP hydrolysis. The first is a highly conserved arginine residue that contacts the γ-phosphate directly (Arg 174 in Gtα) and the second is a glutamine residue (Gln 203 in Gtα) that is positioned to act as a general base to activate the water molecule by proton abstraction. Although heterotrimeric α subunits possess a significantly higher GTPase rate than the small GTPases such as Ras, their deactivation probably requires other proteins. In fact, the turn-off of G protein signalling pathways in vivo occurs 10–100-fold faster than the rate of GTP hydrolysis in vitro, suggesting the existence of proteins able to increase GTP hydrolysis and to return the α subunit to its inactive state. Recently, a family of GTPase activating proteins termed RGS (for regulators of G protein signalling), that deactivates G proteins by allowing inactive heterotrimers to reform, has been identified (Hunt et al., 1996; Watson et al., 1996).

The active GTP-bound α subunit or the released $\beta\gamma$ heterodimer regulate specific downstream effectors. Al-

Table 1
G protein subunit targets of activating mutations in human diseases

G-protein subunit	Intracellular effector	Mutation	Disease
			Gain of function
αs	↑Adenyl cyclase ↑Ca^{2+} channels	Arg 201 > Cys (His/Ser) or Gln 227 > Arg (Leu)	McCune–Albright syndrome GH-secreting pituitary adenoma Nonfunctioning pituitary adenoma ACTH-secreting adenoma Toxic thyroid adenoma Papillary thyroid carcinoma
			Gain and loss of function
		Ala 366 > Ser	Pseudohypoparathyroidism 1a and testotoxicosis
αq	↑Phospholipase C	Not found	
			Gain of function
αi2	↓Adenylyl cyclase ↑K+, ↓Ca^{2+} channels	Arg 179 > Cys (His) or Gln205 > Arg	Nonfunctioning pituitary adenoma, adrenal and sex cord tumors

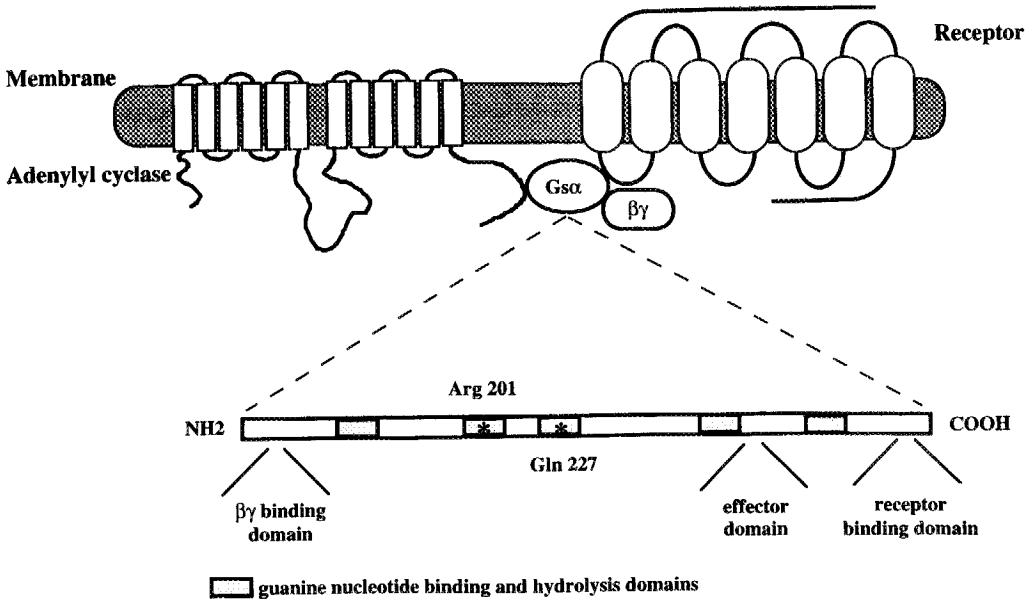

Fig. 1. Schematic representation of Gsα protein domains. The NH_2 terminus is the site of the $\beta\gamma$ binding while critical regions in the COOH terminus specify the α subunit binding to the serpentine receptor. A large domain where Arg 201 and Gln 227 are located is involved in GTP binding and hydrolysis. The Gα surface loops are implicated in adenylyl cyclase binding and activation.

though the family of Gα subunits includes proteins with different functions, unequivocal assignment of one G protein to a single effector molecule has been only achieved for some G proteins. To date, ~20 distinct α subunits have been cloned: according to structural relationships, they can be divided into four major subfamilies represented by Gsα, Giα, Gq/11α and G12α (Table 1). The proteins of the Gs class have been defined as activators of adenylyl cyclase whereas the members of the Gi class, which includes several protein substrates for pertussis toxin ADP ribosylation such as Gi1-3 and Go, are involved in adenylyl cyclase inhibition, ion channel modulation, and phosphatase activation. The subunits of the Gq/11 class are insensitive to pertussis toxin and are putative mediators of phospholipase C activation, whereas the current knowledge about Gα12, 13 is sparse. While prominence was originally given to the α subunit as the pathway for downstream regulation, very active roles for the $\beta\gamma$ subunits have emerged in recent years ((Dhanasekaran et al., 1995; van Biesen et al., 1996). This pathway has been clearly delineated by the demonstration of direct effects of $\beta\gamma$ subunits on different effectors such as K^+ channels, adenylyl cyclase and phosphatidyl inositol specific phospholipase C. Moreover, $\beta\gamma$ subunits appear to be able to transduce mitogenic signals in some cell systems, by regulating Ras activation (Feig, 1994; Dhanasekaran et al., 1995; van Biesen et al., 1996).

Initial experiments and models implicated the Gsα as the primary activating agent of adenylyl cyclase, the enzyme that converts Mg^{2+}-ATP to cyclic AMP and pyrophosphate. Indeed, Gsα stimulates all known isoforms of adenylyl cyclases whereas other regulators, such as $\beta\gamma$, Ca^{2+}/calmodulin, and protein kinases A and C, differentially interact with specific isozymes. Moreover, several lines of evidence obtained in atrial, cardiac sarcolemmal and skeletal muscle T tubule membranes, indicates that Gsα is also involved in positively modulating the activity of voltage-gated dihydropyridine-sensitive Ca^{2+} channels either via a direct action or by cAMP dependent phosphorylation. Finally, Gsα increases intracellular calcium levels by inhibiting inwardly rectifying K^+ channels (Dhanasekaran et al., 1995; Schreibmayer et al., 1996).

The human gene for Gsα (*GNAS*1) has been located on chromosome 20q13.2 > q13.3, contains 13 exons and spans 20 kb (Gejman et al., 1991). The region of chromosome 20 occupied by *GNAS*1 is homologous to an area of mouse chromosome 2 involved in both maternal and paternal imprinting (Williamson et al., 1996). In fact, mice carrying maternal duplication/paternal deficiency for distal chromosome 2 or the reciprocal genotype exhibit phenotypic anomalies. Recent studies have shown that *Gnas*, the mouse homologue of *GNAS*1, lies within this imprinting region, a major difference between the two species being that this gene appears to be paternally imprinted in humans and maternally imprinted in mice. Interestingly, mouse Gsα gene imprinting occurs specifically at the kidney level, consistent with the hypothesis that loss of Gsα expression in renal tubules might be responsible for the resistance to PTH in Albright hereditary osteodystrophy (Williamson et al., 1996).

Four distinct mRNA species are produced by alternative splicing, giving rise to two Gsα proteins with apparent molecular weights of 45 kDa (short form) and two Gsα proteins with apparent molecular weights of 52 kDa (long form). These alternatively spliced forms of Gsα are expressed in a tissue specific distribution. Although the short and long forms of Gsα show subtle differences in biochemical characteristics, such as GTP binding and hydrolysis, receptor coupling and effector activation, none of these differences appears to be physiologically relevant.

3. Gsα and cAMP pathway

Gsα functions by coupling a number of membrane receptors, characterized by a seven transmembrane structure and referred to as serpentine receptors, to one or more isoforms of adenylyl cyclase to generate cAMP. The subsequent activation of cAMP dependent protein kinase A mediates most of the effects of cAMP, that involve biological phenomena as diverse as metabolic and secretory pathways, differentiation and cell growth. As far as regulation of gene expression is concerned, it is well established that cAMP response element binding protein (CREB), once phosphorylated by protein kinase A, can act in the nucleus to modulate the transcription of cAMP-responsive genes by binding to cAMP response elements (CRE) (Fig. 2).

The role of cAMP in regulating cell proliferation is complex and apparently cell type specific. Until recently, the transduction of extracellular signals with mitogenic potential was thought to involve the growth factor receptor tyrosine kinase pathway and the phosphatidyl-inositol protein kinase C cascade. However, recent evidence suggest that in several cell types, particularly epithelial cells, the cAMP cascade positively regulate cell proliferation (Dumont et al., 1989; Maenhaut et al., 1991; Spada et al., 1992). The targets of CREB relevant to mitogenesis include a number of genes that are common to all pathways involved in cell progression to replication, such as the early immediate genes c-*fos*, c-*jun* and *jun* B (Gaiddon et al., 1994). Other events associated with the mitogenic action of cAMP include the increase in Ras and cycline synthesis (Dumont et al., 1989; Zachary et al., 1990). In addition to common genes, cAMP modulates the transcription of genes involved in specific programmes of cell differentiation. Among these, particular attention has been paid to the pituitary specific transcription factor Pit-1, that is required for growth and differentiation of somatotrophs, lactotrophs and thyrotrophs (Chen et al., 1990; Castrillo et al., 1991). Indeed, at variance with the phenotype induced by the activation of the classical oncogenes, the specific pathways activated by cAMP stimulate both growth and specialized functions.

A positive mitogenic effect of cAMP is missing in many cell systems since several lines of evidence indicate that cAMP may either not influence or inhibit

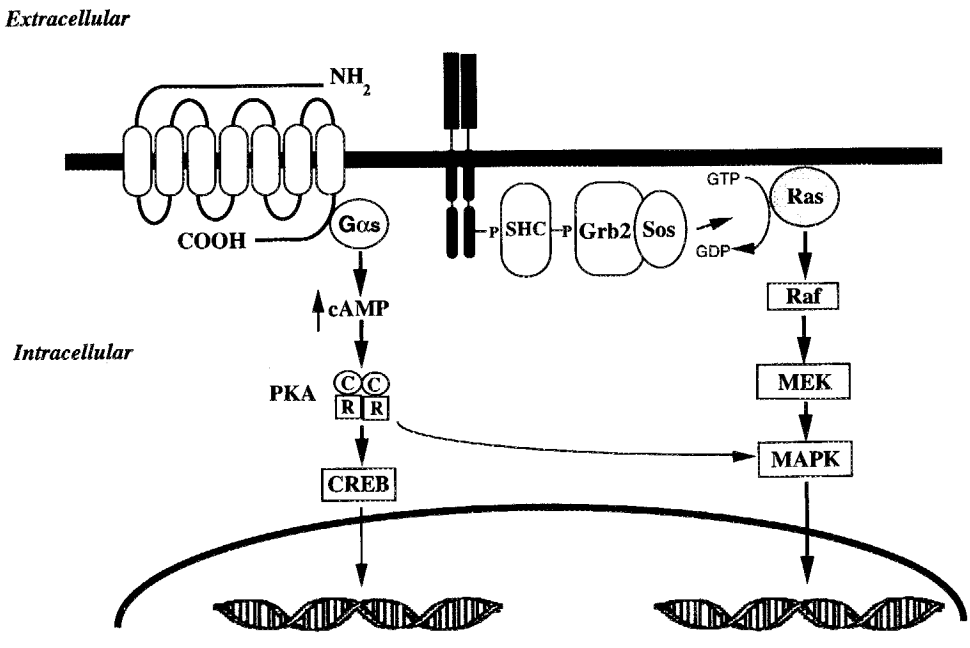

Fig. 2. Role of cAMP in regulating cell proliferation and growth. In specific cell types cAMP, via protein kinase A activation (PKA) and CREB phosphorylation, induces the transcription of a variety of genes involved in mitogenesis. In contrast, in particular cell types, the increased intracellular cAMP levels may inhibit the MAPK cascade triggered by the activation of growth factor receptors.

growth, or even suppress the mitogenic action of growth factors. One of the major signalling pathways controlled by growth factors is the mitogen-activated protein kinase (MAPK) cascade (Fig. 2) (Marshall, 1994). The receptor autophosphorylation induced by growth factor binding recruits adaptor proteins such as Shc and Grb2 that in turn bring the Sos guanine nucleotide exchange factor to catalyze the exchange of GDP for GTP on Ras, leading to its activation. Ras increases the kinase activity of Raf that stimulates MAPK kinase (MEK). Several cell systems treated with cAMP raising agents show decreased responsiveness to specific growth factors, reflecting reduced activation of MAPK pathway (Cook and McCormick, 1993). In these cells, increased cAMP levels are associated with increased phosphorylation of Ser residues in the Raf regulatory domain, resulting in reduced affinity of Raf for Ras. However, in many cell types MAPK pathway is probably not as dominant in the transforming process and the phenotypic effects of PKA activation will be less dramatic. Moreover, it has been observed that in cell systems in which cAMP acts as a mitogen signal cAMP does not reduce or even stimulate MAPK activity, suggesting the existence of a pathway of kinase activation independent of Ras and therefore not susceptible to cAMP inhibition (Faure and Bourne, 1995).

Further experimental evidence is required to identify the mechanisms by which cAMP triggers proliferative or antiproliferative signals in specific cells. Nonetheless, the impact of cAMP pathway dysregulation on cell growth and function has been recently emphasized by the realization that naturally occurring activating mutations of both Gsα and Gs-coupled receptors are associated with tumor formation in humans.

4. Mutations constitutively activating G protein signalling pathways

It has long been known that proteins involved in signalling pathways are possible targets for mutations. As first demonstrated for nuclear hormone receptors and growth factor receptors, it has been proposed that components of G protein signalling pathways may have a latent potential to participate in processes leading to tumor growth (Shenker, 1995; Spiegel, 1996). In the past few years it has been demonstrated that mutations of these proteins, resulting in activation of G protein signalling pathways, are responsible for human diseases presenting with the clinical phenotype of hormone hypersecretion and differentiated neoplasia. The abnormal transduction may be due to mutations in the genes encoding either G protein or G protein coupled receptors. By contrast, mutations of effector molecules seem to occur very infrequently in human diseases. In particular, point mutations of the protein kinase C-α gene leading to amino acid substitution have been reported in invasive human pituitary tumors (Alvaro et al., 1993). However, this abnormality has not been confirmed by subsequent screening studies (Schiemann et al., 1997).

5. Mutations of G protein-coupled receptors in pituitary tumors

Mutations of G protein-coupled receptors may cause gain of function, since certain amino acid substitutions facilitate receptor transition from an inactive to an active conformation in the absence of the ligand. Among the different G protein-coupled receptors that may be target for gain of function mutations, the TSH receptor seems to be involved with the highest frequency (Parma et al., 1993, 1997). In fact, recent screening studies carried out on toxic thyroid adenomas indicate that 70–80% of these neoplasia express mutations in TSH receptor gene (Parma et al., 1997). As it occurs in other G protein-coupled receptors, the majority of these mutations are clustered in the carboxyl half of the third intracellular loop and in the sixth and seventh transmembrane domains, regions involved in G protein coupling and activation. These substitutions cause constitutive activation of the receptor and its associated Gs protein with persistent stimulation of the cAMP dependent pathway. Since cAMP is a mitogenic signal for thyrocytes this probably contributes to tumor formation (Maenhaut et al., 1991).

During recent years screening studies have been carried out on functioning and nonfunctioning pituitary adenomas to identify mutations of serpentine receptors expressed in the pituitary. In fact, considering the crucial role exerted by hypothalamic neurohormones in controlling pituitary cell differentiation, growth and function, several of these receptors seem to be good candidates for oncogenic mutations (Spada et al., 1994; Shimon and Melmed, 1997). However, in GH-secreting adenomas no mutations were found in the GHRH receptor gene, while some adenomas expressed an alternatively spliced truncated receptor unable to transmit GHRH signals (Hashimoto et al., 1995). Moreover, studies of the TRH receptor structure in a large series of tumors, including GH-, PRL-, TSH-secreting adenomas and nonfunctioning adenomas, showed normal wild-type sequence in all tumors (Faccenda et al., 1996). Similarly, analysis of the third exon of the GnRH receptor gene encoding the third intracellular loop and the sixth transmembrane domain that represent the hotspots for activating mutations in other serpentine receptors, revealed no mutations in the 18 gonadotropinomas screened (Kaye et al., 1997). As far as inactivating mutations of receptors that transduce inhibitory signals are concerned, no dopamine D2 re-

ceptor gene mutations were detected in prolactinomas, TSH-secreting adenomas and nonfunctioning adenomas (Friedman et al., 1994). Similarly, no mutations in the somatostatin receptor SSTR2 gene have been identified in GH-secreting adenomas selected on the basis of abnormal in vitro responsiveness to the peptide (Chen et al., 1997). Therefore, based on mutational analyses of several cell surface receptors for hypothalamic releasing and inhibitory factors, there are no current data to support their role in pituitary tumor pathogenesis.

6. Mutations of G proteins in pituitary adenomas

In recent years, molecular biological approaches have provided important insights into the pathogenic role of mutations naturally occurring in G protein genes that alter signal transduction (Spada et al., 1994; van Biesen et al., 1996; Spiegel 1996). At present, Gsα gene is the only G protein gene that has been identified as target for mutations that unequivocally lead to the clinical phenotype of hormone defect or excess (Table 1). The phenotypic expression of these mutations depend on several determinants. Mutations may cause loss of function of Gsα leading to the clinical phenotype of pseudohypoparathyroidism 1α, characterized by resistance to hormones acting through Gs coupled receptors (Patten et al., 1990; Schwindinger et al., 1994; Wilson and Trembath, 1994; Warner et al., 1997). These inactivating mutations are germ-line, and since Gsα is a ubiquitous protein the variable manifestations of the disease probably depend on several factors, including Gsα imprinting (Williamson et al., 1996).

Activating mutations of the Gsα gene so far identified are somatic events, and therefore the mutant protein is only present in a given tissue. The first clue of the possible existence of gain of function mutations in the Gs protein gene naturally occurring in human diseases arose from the identification of a subset of GH-secreting adenomas characterized by high levels of in vitro GH release, intracellular cAMP accumulation and membrane adenylyl cyclase activity (Vallar et al., 1987). The presence of a constitutive activation of Gsα was hypothesized on the basis of the high adenylyl cyclase levels in basal conditions which were not further stimulated by agents directly activating Gsα, such as AlF4 ion and GTP analogues, as well as by specific peptides which operate through Gs coupled receptors, such as GHRH (Vallar et al., 1987). DNA analysis from these tumours by PCR and direct sequencing revealed amino acid substitutions at Arg 201 or Gln 227 in the Gsα gene (Landis et al., 1989; Clementi et al., 1990; Lyons et al., 1990). These mutations were somatic, since only the wild type Gsα was detected in leukocytes from affected patients and heterozygous, as indicated by the presence of both mutant and wild type Gsα in genomic DNA from the tumors (Fig. 3). Conversely, cDNA amplified by PCR revealed that the large majority of the Gsα transcripts originate from the mutant gene. Although it is not known which mechanisms are responsible for this preponderant expression of the mutant Gsα at the mRNA level, this pattern of expression is consistent with the complete unresponsiveness of adenylyl cyclase to AlF-4 ion and GTP analogues (Vallar et al., 1987).

Subsequently, several screening studies confirmed that ≈30–40% of GH-secreting adenomas are associated with these mutations (Landis et al., 1990; Spada et al., 1990; Harris et al., 1992; Adams et al., 1993). A considerably low prevalence (< 5%) has been reported in Japanese acromegalic patients, suggesting a racial difference in the occurrence of the mutation (Hosoi et al., 1993). However, a recent study indicates that Gsα mutations occur in Korean patients with a frequency similar to that observed in western countries (Yang et al., 1996). Gsα mutations have been described with a low prevalence (0–13%) in nonfunctioning pituitary adenomas (Tordjman et al., 1993; Williamson et al., 1994, 1995) while a single study reports Gsα mutations in 5% of ACTH-secreting adenomas (Williamson et al., 1995).

In subsequent studies, mutations involving the same two residues in Gsα gene were identified in hyperfunc-

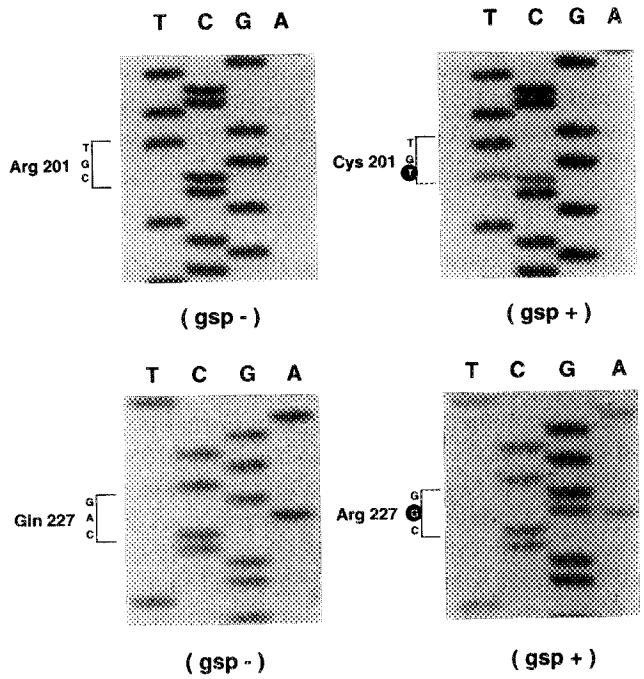

Fig. 3. DNA analysis by polymerase chain reaction and direct sequencing of the Gsα gene from GH secreting tumors. Mutations replace either Arg 201 (wild type codon CGT) with Cys (TGT) or Gln 227(wild type codon CAG) with Arg (CGG) in ~30–40% of GH-secreting adenomas (gsp$^+$). Genomic DNA from gsp$^+$ tumors also show wild type sequence at the same position, indicating heterozygosity of the mutations.

tioning thyroid adenomas (O'Sullivan et al., 1991; Suarez et al., 1991). The prevalence of these mutations, although variable from one series to another, seems to be definitely lower than that of mutations in the TSH receptor gene (Parma et al., 1997). Therefore, the main alterations that constitutively activate the cAMP pathway in thyrocytes are mutations in the TSH receptor gene, while in somatotrophs there are mutations in the Gsα gene. Moreover, Gsα mutations were also identified in a subset of differentiated thyroid adenocarcinomas but not in undifferentiated adenocarcinomas (Suarez et al., 1991).

Evidence suggests that activation of the cAMP pathway may be associated with hyperfunction and hyperplasia of cells other than pituicytes and thyrocytes. In fact, replacement of Arg 201 within the Gsα molecule has been documented in the hyperplastic tissues of patients affected with the McCune–Albright syndrome. This syndrome is characterized by polyostotic fibrous dysplasia, hyperpigmentation and autonomous hyperfunction of several endocrine glands, such as the gonads, the pituitary, the thyroid and the adrenal cortex (Weinstein et al., 1991; Schwindinger et al., 1992). This mosaic distribution is consistent with the idea that this syndrome is due to a somatic mutation in the Gsα gene occurring as an early postzygotic event. Therefore, mutations occurring late cause focal disease such as acromegaly and toxic thyroid adenomas, while when they occur very early in embryogenesis they cause disorders with widespread manifestations, such as the McCune–Albright syndrome. It is tempting to speculate that activating germ-line mutations of Gsα would be incompatible with life (Spiegel, 1996). It is however important to note that, with the exception of pituitary and thyroid adenomas, the endocrine adenomas involved in the McCune–Albright syndrome do not harbour Gsα mutations when they occur sporadically (Yoshimoto et al., 1993).

It should be mentioned that most screening studies have only amplified and sequenced regions encompassing Arg 201 and Gln 227 in the Gsα gene and would not have detected the presence of mutations at sites distant from these residues. However, although in vitro mutagenesis studies have documented a number of possible activating substitutions, all GH secreting adenomas characterized by a high adenylyl cyclase activity not further stimulated by any agents showed substitutions in either Arg 201 or Gln 227, suggesting that other mutations would be relatively infrequent.

7. Type of Gsα mutations and functional studies

Contrary to what was observed for G protein-coupled receptors in which different residues are a possible target for activating mutations (e.g. at least 12 different locations in TSH receptor gene (Parma et al., 1997)), the activating mutations of the Gsα gene so far identified always replace Arg at codon 201 in exon 8 or Gln at codon 227 in exon 9. Point mutations convert Arg 201 to Cys or His or Ser, or Gln 227 to Arg or Leu (Landis et al., 1989; Clementi et al., 1990; Lyons et al., 1990). Mutations most frequently observed replaces Arg 201 (wild-type codon TGC) with Cys (mutant codon TGT). Arg 201 is the only location found in the McCune–Albright syndrome (Weinstein et al., 1991; Schwindinger et al., 1992). Mutations at either Arg 201 or Gln 227 were proved to produce proteins with very low GTPase activity (Landis et al., 1989). Mutations were introduced into a normal rat Gsα cDNA by site-directed mutagenesis and mutant Gsα DNAs, subcloned into the retroviral expression vector MV7 and subsequently transfected into Gsα deficient S49 cyc- cells. Approximately 30-fold decrease in intrinsic GTPase activity was observed in cells expressing Arg201Cys and Gln227Arg mutant proteins (Landis et al., 1989). Indeed, both amino acid residues replaced by these mutations were already known to be important in GTP hydrolysis (Landis et al., 1989; Bourne et al., 1990). Arg 201 is the residue that is ADP-ribosylated by endogenous ADP-ribosyl transferases or bacterial toxins. In particular, the toxin of *Vibrio cholerae* by transferring ADP-ribose from NAD^+ to the side chain of Arg 201 eliminates the intrinsic GTPase activity of Gsα, leading to maintained and hormone-independent activation of adenylyl cyclase. In vitro mutagenesis experiments confirmed that Arg 201 is a key component of the regulatory turn-off mechanism of Gsα, and a similar role is played by Arg residues at equivalent positions in other G proteins, such as Arg 174 in Gtα and Arg 179 in Gi2α. Residue Gln 227 is located in a Gsα region involved in determining the intrinsic GTPase activity of the protein. This Gln corresponds to amino acid 61 in Ras protein, where amino acid substitutions have been shown to inhibit GTP hydrolysis and to result in cellular transformation (Barbacid, 1987).

In principle, in addition to mutations that regulate the ability of the protein to hydrolyze GTP, activating mutations could mimic the normal effect of the hormone–receptor complex, which activates Gsα by increasing the rate at which GDP dissociates from its inactive form. A mutation replacing Ala 366 by Ser in the Gsα gene and accelerating the release of GDP has been described in two unrelated males affected with pseudohypoparathyroidism 1α and gonadotropin independent precocious puberty (Iiri et al., 1994). The mutant protein is rapidly degraded at the body temperature of 37°C, while it is constitutively activated at 32°C (Iiri et al., 1994). Such differential activity may account for the gain of function of the mutant Gsα in the testis, where low temperature prevails, associated with the loss of function in other tissues, particularly in

renal tubules that are target sites for PTH. Therefore, due to the thermolability of the mutant protein, this type of mutation, although able to activate the GTP cycle, does not lead to tumour formation.

8. Mitogenic effects of Gsα mutations

Several lines of evidence indicate that somatotrophs belong to a set of cells that recognize cAMP as a mitogenic signal. In fact, it has been demonstrated that GHRH promotes somatotroph proliferation in vitro and this effect is totally dependent on the cAMP increase induced by the peptide (Billestrup et al., 1986). The proliferative role of GHRH has been confirmed in in vivo models. In fact, in GHRH transgenic mice, pituitaries usually show somatotroph hyperplasia that progress to adenomas in old animals (Asa et al., 1992) while missense mutations in the GHRH receptor reduce the number of somatotrophs in *lit/lit* dwarf mice (Lin et al., 1993). Moreover, the observation that the targeted expression by cholera toxin produces gigantism in transgenic mice further supports the idea that somatotrophs proliferate in response to high cAMP levels (Burton et al., 1991). The role of the cAMP pathway is confirmed by the observation that in transgenic mice the targeted expression of a dominant negative mutant of CREB blocks the proliferation of somatotrophs (Struthers et al., 1991).

In addition to somatotrophs, activation of the cAMP cascade is a mitogenic signal for thyrocytes, and this idea has been supported by results obtained in transgenic animals (Dumont et al., 1989; Maenhaut et al., 1991). In fact, targeting the expression of the Gs-coupled A2 adenosine receptor to thyroid cells of transgenic mice causes thyroid tumors (Ledent et al., 1992). Similarly, expression of mutant Gsα transgene directed to thyroid cells by thyroglobulin promoter results in development of hyperfunctioning thyroid adenomas (Michiels et al., 1994). Due to the mitogenic role of the cAMP pathway in somatotrophs and thyrocytes, Gsα protein may be considered the product of a proto-oncogene that is converted into an oncogene, designated gsp (for Gs protein) in selected cell types.

Recently, molecular biology studies have been carried out to test the effects of mutant Gsα on gene transcription. The mutant protein was found to strongly increase transcription of a variety of early immediate genes, including c-*fos*, c-*jun* and *jun* B in GH3 and AtT20 pituitary cells, consistent with the idea that the expression of these nuclear proteins is modulated by the cAMP pathway (Montminy et al., 1990; Gaiddon et al., 1994). Recently, it has been demonstrated that mutant Gsα potently stimulates CREB phosphorylation and cAMP response element (CRE)-dependent promoter activity in human adenomatous somatotrophs (Bertherat et al., 1995). In that study, 5–10-fold higher levels of wild type Gsα in comparison with mutant protein were necessary to induce similar stimulation of CREB activity, suggesting that Gsα gene may promote somatotroph transformation by inducing transcription of specific CREB-dependent target genes (Bertherat et al., 1995). Indeed, mutant Gsα was proven to strongly stimulate transcription directed by both the human PRL and GH gene promoters (Gaiddon et al., 1995).

9. Phenotype of cells transfected with mutant Gsα; role of phosphodiesterase system

The phenotype resulting from expression of mutant Gsα has been investigated in cells in which the cAMP cascade enhances function and proliferation, such as Swiss 3T3 fibroblasts, FRTL-5 thyroid cells and GH3 pituitary cells (Zachary et al., 1990; Muca and Vallar, 1994; Ham et al., 1997). The introduction of the Gln227Leu mutation in FRTL-5 cells is sufficient to induce a TSH independent proliferation, although with a growth rate slower than that observed under TSH stimulation (Muca and Vallar 1994). Similarly, GH3 cells expressing the same mutant Gsα show enhanced proliferation and hormone (GH and PRL) secretion. In Swiss 3T3 cells carrying mutant Gsα, the mitogenic activity is higher than that of wild type cells, as indicated by the low serum concentration required for growth (Zachary et al., 1990). However, in these cells the activated phenotype was observed only when cAMP hydrolysis was blocked by inhibitors of phosphodiesterase (PDE). Similarly, expression of the Arg201Cys mutation in pancreatic β cells of transgenic mice caused the anticipated increase in insulin secretion only when cAMP degradation was blocked (Ma et al., 1994).

A given cAMP concentration is the result of a steady state of synthesis and degradation which is catalyzed by a large number of different PDE isoenzymes (Conti et al., 1995). The PDE activity is finely regulated by modification of intracellular cAMP levels. In particular, increased cAMP levels induce a short term activation of cAMP specific-PDEs (PDE4) via a series of phosphorylation processes and a long-term activation via gene expression and protein synthesis regulation (Conti et al., 1991, 1995) (Fig. 4). Studies carried out in FRTL-5 cells demonstrated that the expression of mutant Gsα is accompanied by a concomitant induction of PDE4, its blockade resulting in a further stimulation of the cAMP level and cell proliferation (Nemoz et al., 1995). Similarly, it has been recently reported that the feedback mechanism by which cAMP controls the expression of its own degrading enzymes is overactive in human GH-secreting adenomas carrying mutant Gsα (Lania et

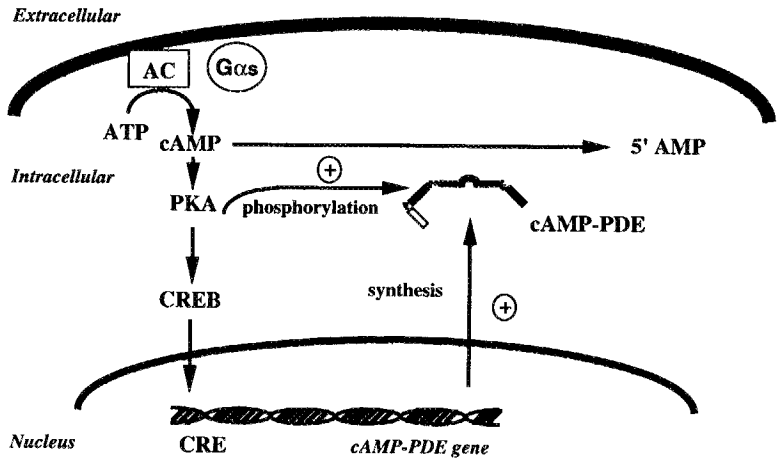

Fig. 4. The cAMP-specific PDE (cAMP-PDE) activity is regulated by intracellular cAMP levels either by phosphorylation processes (short term activation) and by a gene expression and protein synthesis regulation (long-term activation). PKA, protein kinase A.

al., 1998). Indeed, inhibition of PDE activity, particularly PDE4, results in a marked increase in cAMP accumulation in cells from gsp$^+$ tumors, suggesting a high rate of cAMP hydolysis in these cells. By direct measurements, in mutant tumors PDE activity resulted to be about 7-fold higher than that observed in wild type tissues (Fig. 5) (Lania et al., 1998). Although the study does not discriminate whether the increase in PDE4 activity is due to an activation or an increased expression of the enzyme, it is reasonable to hypothesize that the long-term adaptation of somatotrophs to constitutively activated cAMP production might require both enzyme activation and increased gene expression. These data suggest that the increased cAMP degradation caused by PDE overactivity may, at least in part, contrast the consequence of the constitutive activation of cAMP pathway and contribute to determine the oncogenic potential of mutant Gsα and the clinical phenotype. It remains to be elucidated whether the PDE feedback loop is also upregulated in other endocrine disorders caused by mutations in Gsα gene, such as thyroid toxic adenomas and the McCune–Albright syndrome.

10. Clinical phenotype of patients with tumors carrying gsp mutations

Over the last few years, screening studies carried out on a large number of GH-secreting adenomas have confirmed that the prevalence of Gsα mutations in acromegalic patients is between 30 and 40% (Landis et al., 1990; Spada et al., 1990; Harris et al., 1992; Adams et al., 1993). The in vivo studies indicate no difference in age, sex, clinical features, duration of the disease or cure rate between patients with and without Gsα muta-

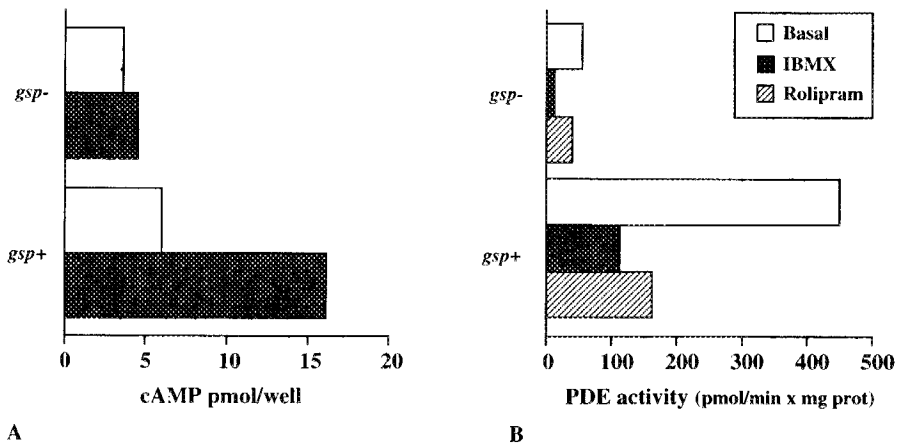

Fig. 5. (A) Intracellular cAMP levels in cultured cells obtained from gsp$^+$ and gsp$^-$ tumors in absence and presence of the non selective phosphodiesterase inhibitor IBMX. (B) PDE activity measured in membrane preparations obtained from tumors with or without Gsα mutations in basal conditions and in presence of either non selective (IBMX) and cAMP-specific PDE selective (rolipram) inhibitors. In gsp$^+$ tumors PDE activity was higher than in gsp$^-$, and was largely due to cAMP-specific PDE.

tions (gsp^- and gsp^+ tumors). Although no striking difference in GH levels was observed, patients with gsp^+ tumors develop full manifestations of the disease in the presence of very small adenomas, consistently with an hypersecretory activity of the tumor (Spada et al., 1990). This was confirmed by the ultrastructure morphology of the tumors, that appeared to be constituted of densely granulated cells with a well-developed secretory apparatus. As anticipated, patients with gsp^+ tumors did not increase plasma GH levels after GHRH administration while they showed secretory responses to agents that activate cAMP independent pathways, such as TRH. Moreover, these patients seem to be extremely sensitive to the inhibitory action of somatostatin which reduces GH release by inhibiting cAMP production (Spada et al., 1990).

Although activating mutations of Gsα would in principle confer growth advantage, patients with gsp^+ tumors do not differ in the rate of tumor growth or recurrency from the other patients, and both groups show similar progression of the disease (Spada et al., 1990). The low rate of tumor growth correlates well with the poor morphological evidence of cell replication in gsp^+ tumors. This in vivo phenotype probably reflects the existence of controregulatory mechanisms upregulated by the constitutive activation of the cAMP pathway, such as a high sensitivity to somatostatin and/or overactivity of the PDE system, both events resulting in a reduction of cAMP accumulation.

As far as the clinical characteristics of the reported nonfunctioning tumors expressing gsp oncogene are concerned, either a gonadotropinoma with high serum FSH levels or tumors immunostaining for LH/FSH or ACTH were identified (Tordjman et al., 1993; Williamson et al., 1994). As reported for GH-secreting adenomas, there was no clinical characteristic differentiating tumors harbouring or not Gsα gene mutations.

11. Mutations of Gi2α subunit in pituitary adenomas

Taking into account that all the G proteins have a common mechanism of binding and hydrolyzing GTP and share highly conserved primary structures in regions corresponding to Arg 201 and Gln 227 of Gsα, it was predicted that other G proteins would be converted into oncogenes by GTPase inhibiting mutations. The screening studies of different types of human tumors for mutations in Gi2α revealed amino acid substitutions in Arg 179, which corresponds to Arg 201 of the Gsα gene, in a proportion of ovarian and adrenal tumors (Lyons et al., 1990) (Table 1). Probably due to the low number of analyzed tumors, the true prevalence of these mutations (referred to as gip 2) is still undefined (Reincke et al., 1993). Recently, mutations at codon 205 of Gi2α resulting in a conversion from Gln to Arg have been identified in three out of 22 nonfunctioning pituitary adenomas (Williamson et al., 1994). Interestingly, two of these tumors also had concomitant gsp mutations, with a paradoxical result in terms of cAMP generation, considering that Gsα and Gi2α have opposing actions on adenylyl cyclase activity.

The oncogenic potential of the constitutive activation of Gi2α is difficult to ascertain since the intracellular pathways activated by Gi2α are multiple and probably not fully understood. The gip 2 oncogene product has been shown to cause constitutive inhibition of adenylyl cyclase and reduction of cytosolic calcium in transfected cells (Pace et al., 1991; Wong et al., 1991). Moreover, the mutationally activated Gi2α activates a growth inhibiting signal by modulating the expression of the tumor suppressor gene Rb (Ikezu et al., 1994). However, a possible role of gip 2 oncogene on mitogenic pathways is suggested by the observation that Gi-coupled receptors also stimulate the MAP kinase cascade (Gupta et al., 1992a). Consistently, in certain cell systems the expression of constitutively activated Giα causes cell transformation (Gupta et al., 1992b).

12. Mutations of other G proteins in pituitary tumors

Other members of the G protein family mediate mitogenic responses. By in vitro mutagenesis it has been recently found that activated mutants of Gqα, G12α, G13α and Gzα are fully oncogenic, G13α being one of the most potent oncogenes so far identified (Xu et al., 1994; Dhanasekaran et al., 1995; Wong et al., 1995). Although these findings indicate that other families of G proteins have potent transforming properties, it remains to be shown whether or not these genetic abnormalities might naturally occur in pituitary adenomas. Recently, fragments of Gqα cDNA encompassing residues Arg 183 and Gln 209 from a large group of pituitary adenomas of all types have been screened and no mutations were detected, suggesting that mutations in these regions of Gqα occur infrequently, if at all, in pituitary adenomas (Oyesiku et al., 1997).

13. G protein expression in pituitary tumors

Abnormal expression of G proteins in different target tissues has been described in numerous pathophysiological states (Spiegel et al., 1992). In particular, it has been proposed that reduced expression of proteins of the Gi family may prevent transduction of inhibitory signals in pituitary tumors (Wood et al., 1991). This phenomenon seems to occur in pituitary cell lines that are refractory to dopamine action, probably due to a lack of G proteins sensitive to pertussis toxin (Cronin et

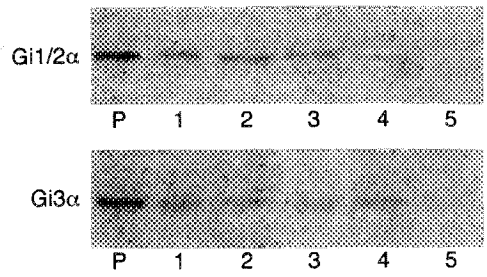

Fig. 6. Representative immunoblotting performed with antibodies to Gi1/2α and Gi3α. Each lane was loaded with 20 μg of crude membrane proteins obtained from normal pituitary (P), GH-secreting (1, 2, 5) and nonfunctioning pituitary adenomas (3 and 4). As clearly indicated, the pituitary adenomas are characterised by a reduced or undetectable (tumor 5) expression of proteins of the Gi family, as compared with the normal pituitary.

al., 1981). Similarly, a selective reduction of Gi2α mRNA has been recently documented in prolactinomas removed from patients resistant to dopaminergic drugs (Caccavelli et al., 1996). Indeed, the reduced expression of proteins of the Gi family, particularly Gi1–3α, seems to be a common event in pituitary adenomas, independently from the nature of the tumor (Ballarè et al., 1997) (Fig. 6). Although the mechanisms responsible for the low expression of these proteins are still unknown, this phenomenon is specific of tumoral pituitary tissues and may contribute to uncontrolled growth. In fact, several observations indicate that both dopamine and somatostatin receptors couple to Gi1/2α to inhibit cAMP formation, while Goα is probably associated to Ca^{2+} channel regulation (Vallar and Meldolesi, 1989; Civelli et al., 1993; Chen et al., 1994; Reisine and Bell, 1995). It is therefore tempting to speculate that, although cells probably require relatively few G proteins for transducing hormonal signals, the low expression of Gi1–3α proteins may be responsible for a poor capacity of inhibitory neurohormones to reduce the production of cAMP, that represents a proliferative signal.

At present, it is not possible to establish whether a low expression of Giα protein is specific to tumors of pituitary origin. However, it has been recently reported that in toxic thyroid adenomas the pattern of G protein expression seems to be different, high levels of Gsα being accompanied by high levels of Giα (Derwahl et al., 1996).

14. Concluding remarks

It is well established that cAMP is involved in the control of metabolic and differentiation processes in a large number of endocrine cells. Moreover, the identification of activating mutations of the adenylyl cyclase stimulatory protein Gsα in human hyperfunctioning tissues suggests a proliferative action of cAMP in selected cell types, such as somatotrophs and thyrocytes. Since cAMP pathway is involved in somatotroph replication, it has been proposed that Gsα gene may be converted into an oncogene, designated *gsp* (for Gs protein) in certain cell types. While in vitro phenotype of somatotrophs expressing these mutations is consistent with the constitutive activation of Gs protein, no significant differences in the in vivo phenotype of acromegalic patients bearing adenomas with or without *gsp* mutations have been so far reported. This observation is in part explainable by the increased PDE activity reported in somatotroph cells characterized by the overactivation of the cAMP pathway. These data suggest that mutant Gsα phenotype may be at least partially reverted by counter-regulatory mechanisms and further strengthen the hypothesis that multiple processes contribute to determine the oncogenic potential of activating mutations of Gsα in the different cell types.

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